Analysis of the Cell Fusion Activities of Chimeric Simian Immunodeficiency Virus-Murine Leukemia Virus Envelope Proteins: Inhibitory Effects of the R Peptide

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It was previously reported that truncation or proteolytic removal of the C-terminal 16 amino acids (the R peptide) from the cytoplasmic tail of the murine leukemia virus (MuLV) envelope protein greatly increases its fusion activity. In this study, to investigate the specificity of the effect of the R peptide on the fusion activity of viral envelope proteins, we expressed simian immunodeficiency virus (SIV)-MuLV chimeric proteins in which the entire cytoplasmic tail of the SIV envelope protein was replaced by either the full-length MuLV cytoplasmic tail or a truncated MuLV cytoplasmic tail with the R peptide deleted. Extensive fusion of CD4-positive cells with the chimeric protein containing a truncated MuLV cytoplasmic tail was observed. In contrast, no cell fusion activity was found for the chimeric protein with a full-length MuLV cytoplasmic tail. We constructed another SIV-MuLV chimeric protein in which the MuLV R peptide was added to an SIV envelope protein cytoplasmic tail 17 amino acids from its membrane-spanning domain. No fusion activity was observed within this construct, while the corresponding truncated SIV envelope protein lacking the R peptide showed extensive fusion activity. No significant difference in the transport or surface expression was observed among the various SIV-MuLV chimeric proteins and the truncated SIV envelope protein. Our results thus demonstrate that the MuLV R peptide has profound inhibitory effects on virus-induced cell fusion, not only with MuLV but also in a distinctly related retroviral envelope protein which utilizes a different receptor and fuses different cell types.

The envelope glycoproteins of retroviruses are important for viral infectivity and play critical roles in the viral life cycle. They are synthesized as precursor proteins which are glycosylated and processed into SU and TM subunits, mediated by a cellular protease (13, 40–42). The SU protein mediates binding to receptors and determines the host range of virus infection (3, 25, 27). The TM protein has three distinct domains: an extracellular domain which interacts with the SU protein and also contains a stretch of about 20 hydrophobic amino acids at its N terminus, which is designated the fusion peptide; a membrane-spanning domain which anchors the envelope protein in the cell and viral membrane; and the cytoplasmic tail.

Lentivirus envelope proteins contain cytoplasmic domains that are very long compared with those of other retroviruses (more than 150 amino acids for lentiviruses versus fewer than 50 amino acids for other retroviruses). Studies with simian immunodeficiency virus (SIV) have shown that after prolonged passage in human cell lines, a virus containing a truncated envelope transmembrane protein is selected (5, 16). However, when SIV clones encoding truncated envelope proteins were used to infect monkey cells, reversion to the full-length envelope protein occurred (16, 19). Furthermore, rhesus macaques infected with SIVmac1A11, which contains a truncated TM protein, developed transient viremia but did not develop a clinical disease, while animals infected with SIVmac239, possessing a full-length TM protein, developed a progressive AIDS-like disease which ultimately resulted in death (18, 23). Further studies showed that truncation of most of the envelope protein cytoplasmic tail of SIVmac239 greatly increases its fusion activity (32, 43). Studies with equine infectious anemia virus also indicated that, depending on the cell lines used for virus propagation, viruses containing full-length or truncated envelope proteins were selectively produced (31). In human immunodeficiency virus type 1 (HIV-1), only one case of an infectious clone possessing a truncated envelope TM protein has been reported (36). The truncation of the TM protein apparently arose by adaptation to TALL-1 cells after prolonged passage (36). The truncation of the TM protein cytoplasmic tail by passage through specific cell lines indicates that the full-length cytoplasmic tail is not strictly required for virus infectivity.

In murine leukemia virus (MuLV), Mason-Pfizer monkey virus, and equine infectious anemia virus, the envelope TM protein cytoplasmic tail is further processed by the viral protease after virus particle assembly (11, 31, 36). In MuLV, this processing of the TM protein produces the mature TM protein p12E and releases a fragment consisting of the C-terminal 16 amino acids, which has been designated the R peptide (11, 14). Recent studies have shown that processing of the R peptide is important for virus infectivity. Mutations at the cleavage site which block processing of the R peptide greatly decreased virus infectivity, and truncation of the R peptide greatly increased the fusion activity of the MuLV envelope protein even in the absence of other viral components (28, 30). These studies suggested that the C-terminal portion of these retrovirus envelope protein cytoplasmic tails functions to modulate viral fusion activity. Interestingly, the cytoplasmic tail of the processed MuLV envelope protein is similar in size to that of the naturally truncated SIV envelope proteins which show enhanced cell fusion activity. In this study, to further investigate the specificity of the effects of the R peptide on viral fusion activity, we constructed chimeric envelope proteins which contained either a full-length or an R peptide-truncated MuLV

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cytoplasmic tail and the transmembrane and external domains of the SIV envelope protein. We have determined the effects of the R peptide on the synthesis, transport, and cell fusion activity of these chimeric envelope proteins.

MATERIALS AND METHODS

Cells and viruses. HeLa and HeLa T4 cells were obtained from the American Type Culture Collection, Rockville, Md. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (GIBCO BRL). The construction of vaccinia virus recombinants which express full-length or truncated SIVmac239 has been described (32). A recombinant vaccinia virus (vTF7-3) expressing the T7 polymerase was provided by R. Moss.

Plasmid construction and mutagenesis. All restriction endonucleases and DNA modification enzymes used for plasmid construction were purchased from Boehringer Mannheim Biochemicals. The BamHI-PvuII (nucleotides 7565 to 7884) fragment from plasmid pMven or MuLVenvR was isolated, blunt-ended by treatment with mung bean nuclease (Boehringer) and inserted at the Nhel site of the SIVmac239 envelope gene construct (SIV239) which had been blunt-ended by filling in with Klenow polymerase. The resulting plasmid constructs express SIV-MuLV chimeric envelope proteins in which the entire SIV envelope protein cytoplasmic tail is replaced by the full-length or truncated cytoplasmic tail of the MuLV envelope protein. The resulting plasmid constructs were designated S-Menv and S-MenvR, respectively.

The plasmid containing a gene encoding an SIV-MuLV chimeric envelope protein in which the R peptide is attached to the C terminus of the truncated SIV envelope protein SIV733T was constructed by the following procedure. The coding sequence of the MuLV R peptide (encoding the C-terminal 18 amino acids of the MuLV envelope protein) was amplified by PCR (Vent polymerase; New England Biolabs) with primers 5'TCTAAGGCTTGACTAGCTTCG3' and 5'AGGTGACACTATAGAATACACG3' encoding the C-terminal 18 amino acids of the MuLV envelope protein were inserted into the BamHI-PvuII (nucleotides 7565 to 7884) fragment from plasmid pMven or MuLVenvR which was isolated, blunt-ended by treatment with mung bean nuclease (Boehringer), and inserted at the Nhel site of the SIVmac239 envelope gene construct (SIV239) which had been blunt-ended by filling in with Klenow polymerase. The resulting plasmid constructs express SIV-MuLV chimeric envelope proteins in which the entire SIV envelope protein cytoplasmic tail is replaced by the full-length or truncated cytoplasmic tail of the MuLV envelope protein. The resulting plasmid constructs were designated S-Menv and S-MenvR, respectively.

Plasmid SIV750T was constructed by site-directed PCR mutagenesis with primers 5'CCACCGTCTCATTATTTGCCTTC3' and 5'GGATAACGCTTAC CAGAAGCCCA3'. The PCR-amplified MuLV envelope gene fragment was cut by BsrGI in which Klenow polymerase was added and cut with Asp718I. This fragment was ligated to the SIV envelope protein gene at the newly introduced PvuII site and the Asp718I site. The resulting construct is designated plasmid SIV733T-R.

RESULTS

Expression and fusion activity of the MuLV envelope protein. To investigate the effect of the R peptide on virus-induced cell fusion, we introduced a stop codon in the MuLV envelope gene by site-directed mutagenesis. The resulting construct expresses an MuLV envelope protein truncated two amino acids before the R peptide cleavage site in the cytoplasmic tail. The amino acid sequences of the MuLV envelope protein and the R peptide truncated mutant are shown in Fig. 1. Expression of wild-type and mutant envelope proteins in the vaccinia virus T7 expression system (9) is shown in Fig. 2. The R peptide-truncated MuLV envelope TM protein migrated faster than

**FIG. 1. Schematic diagram of the envelope glycoproteins used in this study.**

The amino acid sequences of the cytoplasmic tail of each envelope glycoprotein are shown in this diagram. The designations of each envelope glycoprotein are shown on the left and are the same as their respective plasmid constructs. Amino acids of MuLV origin are underlined. Sequences in open boxes represent the R peptide. Shaded boxes represent transmembrane regions of these envelope glycoproteins.

**FIG. 2. Expression and fusion activity of MuLV envelope proteins.** The fusion activities of the SIV and chimeric SIV-MuLV envelope proteins were analyzed by the following procedure. HeLa T4 cells seeded into six-well plates and grown to 80% confluence were infected with vTF7-3 and transfected with plasmid constructs containing genes encoding the SIV envelope protein or the SIV-MuLV chimeric envelope proteins by using Lipofectin (GIBCO BRL) as described above for protein expression. At 12 h postinfection, infected and transfected HeLa T4 cells were detached from plates by treatment with 2 mM EDTA and suspended in RPMI 1640 plus 10% fetal calf serum. They were then mixed with Hut 78 cells and dispensed into individual wells of 24-well tissue culture plates. Cell fusion was monitored and photographs were taken with a Nikon phase-contrast microscope after 6 to 8 h.

Biotinylation of surface proteins. The surface expression of each construct was assayed by a surface biotinylation assay (38). HeLa cells were seeded, infected, and transfected as described above. At 12 h postinfection, cells were starved with Eagle's medium deficient in methionine and cysteine for 45 min and then pulse labeled with 150 μCi of [35S]Met/Cys (NEN, Du Pont) in 800 μl of methionine and cysteine-deficient DMEM for 45 min. After labeling, cells were washed once with DMEM plus 10% bovine calf serum and then chased in DMEM plus 10% bovine calf serum. After 4 h of chase, the cells were washed three times with phosphate-buffered saline (PBS), biotinylated at 4°C for 30 min with 0.5 mg of NHS-S-S-Biotin (Pierce) per ml in PBS, lysed with lysis buffer, and then immunoprecipitated with appropriate antibodies and protein A-agarose at 4°C overnight. Samples were washed twice in lysis buffer plus 0.4% SDS and then split into two equal portions. One portion was mixed with 15 μl of reducing sample buffer and used to analyze total cellular expression. The other portion was mixed with 20 μl of 10% SDS and heated at 95°C for 15 min in order to dissociate the complex. Dissociated proteins were then dissolved in 700 μl of lysis buffer and incubated with 10 μl of streptavidin-agarose (Pierce) for 5 h at 4°C. Biotinylated samples were washed three times with lysis buffer, mixed with 15 μl of reducing sample buffer, and heated at 95°C for 5 min before they were loaded onto SDS-PAGE gels.

**RESULTS**

Expression and fusion activity of the MuLV envelope protein. The fusion activities of MuLV envelope constructs were determined by the following procedure. HeLa T4 cells which had been seeded into six-well plates the previous day and grown to 80% confluence were infected with vTF7-3 and transfected with plasmid constructs containing wild-type or mutant MuLV envelope genes by using Lipofectin

(GIBCO BRL) as described above for protein expression. At 12 h postinfection, they were overlaid with XC cells, a transformed rat cell line. Cell fusion was monitored 6 to 8 h later, and photographs were taken with a Nikon phase-contrast microscope.

Fusion assay of SIV and chimeric proteins. The fusion activities of the SIV and chimeric SIV-MuLV envelope proteins were analyzed by the following procedure. HeLa T4 cells seeded into six-well plates and grown to 80% confluence were infected with vTF7-3 and transfected with plasmid constructs containing genes encoding the SIV envelope protein or the SIV-MuLV chimeric envelope proteins by using Lipofectin (GIBCO BRL) as described above for protein expression. At 12 h postinfection, infected and transfected HeLa T4 cells were detached from plates by treatment with 2 mM EDTA and suspended in RPMI 1640 plus 10% fetal calf serum. They were then mixed with Hut 78 cells and dispensed into individual wells of 24-well tissue culture plates. Cell fusion was monitored and photographs were taken with a Nikon phase-contrast microscope after 6 to 8 h.
thefull-lengthMuLVenvelopeprotein(comparelane2with
lane3).Also,thetruncatedproteincouldnotbeprecipitated
by antibodies generated against the R peptide (data not
shown).ThetruncatedMuLVenvelopeproteinlackingtheR
peptideshowedlevelsofsecretionandsurfaceexpressionas
well as processing to the SU and TM proteins similar to those
of the full-length MuLV envelope protein. These results
showed that truncation of the R peptide does not affect the
expression, transport, or surface expression level of the MuLV
envelope proteins.

To determine the effects of the R peptide on fusion activity,
we expressed wild-type and truncated MuLV envelope pro-
teins in HeLa T4 cells with the vaccinia virus T7 system and
cocultivated them with XC cells, a transformed rat cell line
which can be fused by MuLV under neutral pH conditions
(24). The results shown in Fig. 3 demonstrate that the protein
with a truncation of the R peptide caused fusion of cells into
large syncyta, while the full-length MuLV envelope protein
exhibited no observable fusion activity. This is in accordance
with the results of other groups using different expression sys-
tems and cell types, who also reported that truncation of the R
peptide greatly increases the fusion activity of the MuLV en-
velope protein (28, 30).

Construction and expression of chimeric SIV-MuLV enve-
lope proteins. Previous studies have shown that passage of SIV
in established human cell lines resulted in truncation of the
SIV envelope protein, leaving 17 amino acids in the cytoplas-
mic tail (5, 16). Furthermore, it has been reported that trun-
cation of the cytoplasmic tail greatly affects its fusion activity in
various cell types and alters the conformation of the extracel-
ular domain (5, 16, 32, 38). Interestingly, the remaining cyto-
plasmic tail of the truncated SIV envelope proteins is about
the same length as the cytoplasmic tail of the processed, mature
MuLV envelope protein. In addition, analysis with a helical
wheel plot shows that in both the truncated SIV and the pro-
cessed MuLV envelope proteins, the remaining cytoplasmic
tail can form an amphipathic helix (Fig. 4). To investigate
whether the presence of the MuLV cytoplasmic tail sequence
could affect fusion activity in proteins with an SIV external and
transmembrane domain, we constructed genes which encode
chimeric SIV-MuLV envelope proteins in which the entire
cytoplasmic tail of the SIV envelope protein was replaced by
either a full-length or truncated cytoplasmic tail of the MuLV
envelope protein, designated S-Menv and S-MenvR⁻, respec-
tively (as shown in Fig. 1).

Figure 5 shows the expression of truncated SIV envelope
proteins and chimeric SIV-MuLV envelope proteins in the
vaccinia virus T7 system. As expected, the S-MenvR⁻ chimera
was similar in molecular weight to SIV733T; they contain 16
and 17 amino acids in their cytoplasmic tails, respectively
(lanes 2 and 4). The S-Menv protein migrates slower than
S-MenvR⁻ and SIV733T, as expected (about 2 kDa higher in
apparent molecular mass), since it contains 33 amino acids in
its cytoplasmic tail (lane 3). Immunoprecipitation with anti-
body against the R peptide showed that the S-Menv chimera
protein with the full-length MuLV cytoplasmic tail can still be
recognized, indicating that the R peptide still maintains its
normal antigenic conformation (data not shown). Also shown
in Fig. 5 are the secretion and surface expression of SIV and
chimeric SIV-MuLV envelope proteins. Similar to the results
with MuLV envelope proteins, the presence or absence of the

FIG. 2. Expression of wild-type and truncated MuLV envelope proteins.
HeLa T4 cells were infected with vTF7-3 and then transfected with plasmids
containing genes encoding wild-type or truncated MuLV envelope protein. At 12
h postinfection, cells were labeled with [35S]Met/Cys as described in Materials
and Methods. After labeling, cells were biotinylated and then immunoprecipi-
tated with antibodies against MuLV plus protein A-agarose beads at 4°C over-
night. The samples were prepared with reducing sample buffer, heated at 95°C
for 5 min, and then loaded onto SDS-PAGE gels. Lanes 1, mock transfection;
lanes 2, MuLVen; lanes 3, MuLVenR⁻.

FIG. 3. Fusion of XC cells by MuLV envelope proteins. HeLa T4 cells were infected with vTF7-3 and transfected as described for protein expression. At 12 h
posttransfection, they were overlaid with XC cells. Fusion was monitored by microscopy, and photographs were taken 8 h later. (a) Mock transfection; (b) MuLVen;
(c) MuLVenR⁻.
R peptide in the cytoplasmic tail does not affect the processing, transport, or surface expression of these chimeric envelope proteins (compare lanes 3 and 4 with lane 2). The higher surface expression level of S-MenvR \(_2\) (lane 4) is probably due to variation in the transfection efficiency, since it also has a higher cellular expression level.

**Fusion activities of chimeric SIV-MuLV envelope proteins.** We further investigated the effect of the R peptide on the fusion activity of the chimeric SIV-MuLV envelope proteins. The envelope proteins SIV\(_{env733T}\), S-Menv, and S-MenvR\(_2\) were expressed in the vaccinia virus T7 system as described in Materials and Methods. At 10 h posttransfection, infected and transfected HeLa T4 cells were detached by treatment with EDTA, mixed with Hut 78 cells, and monitored for fusion activity. As shown in Fig. 6, the S-MenvR\(_2\) protein, which lacks the R peptide, induces extensive cell fusion, similar to SIV\(_{733T}\). However, the S-Menv chimeric protein, with a full-length MuLV cytoplasmic tail, showed no detectable fusion activity. Similar results were also obtained when other types of CD4-positive cells, such as CEMx174 cells and H9 cells, were used (not shown). This result indicates that a chimeric molecule with an SIV external domain and a full-length MuLV envelope protein cytoplasmic tail is defective in inducing cell fusion, whereas elimination of the MuLV R peptide results in a highly fusogenic protein. This is interesting because the SIV-MuLV chimeric envelope protein utilizes a different receptor from that of the MuLV envelope protein and fuses different cell lines.

**Fusion activity of a truncated SIV envelope protein is inhibited by the presence of the R peptide.** To further investigate the molecular requirements for the inhibitory effect of the R peptide on the fusion activity of chimeric SIV-MuLV proteins, we constructed a plasmid encoding chimeric protein SIV\(_{env733T}\)+R, in which the R peptide was attached to the C terminus of an SIV envelope protein with a 17-amino-acid cytoplasmic tail. For comparison, we also constructed a gene encoding a truncated SIV envelope protein, SIV\(_{750T}\), which contains 33 SIV-derived amino acids in its cytoplasmic tail and is therefore similar in length to the uncleaved MuLV envelope protein cytoplasmic tail (see Fig. 1). Figure 5 shows the expression of these two constructs in the vaccinia virus T7 system in HeLa T4 cells (lanes 5 and 6). The addition of the R peptide or 16 additional SIV amino acids to SIV\(_{733T}\) apparently does not affect the processing, transport, or surface expression of these envelope proteins. When analyzed for cell fusion activity, as shown in Fig. 7, the SIV\(_{750T}\) protein was found to induce extensive cell fusion, while the SIV\(_{733T}+R\) molecule does not induce any detectable cell fusion. These results demonstrate that the presence of the R peptide has a dramatic inhibitory effect on the fusion activity of the truncated SIV envelope.
protein, whereas a sequence of similar length derived from SIV had no inhibitory effect. Thus, the inhibitory effect of the MuLVR peptide on virus-induced cell fusion appears to be a reflection of its specific amino acid sequence.

**DISCUSSION**

In this study, we investigated the effects of specific alterations in the cytoplasmic tail on the fusion activity of the MuLV envelope protein and of chimeric SIV-MuLV envelope proteins. In agreement with other studies which utilized different expression systems (28, 30), we observed that deletion of the R peptide greatly increased the fusion activity of the MuLV envelope protein. We also found that the R peptide had a similar inhibitory effect on the fusion activity of chimeric SIV-MuLV envelope proteins with MuLV cytoplasmic tails. Moreover, we found that addition of the MuLV R peptide to the cytoplasmic tail of a highly fusogenic truncated SIV envelope protein resulted in inhibition of its fusion activity as well. These results indicate that the R peptide has significant inhibitory effects on the cell fusion activity of both the MuLV envelope protein and chimeric SIV-MuLV envelope proteins.

The function of the cytoplasmic tails of retroviral envelope proteins in virus replication is not well understood. Studies with HIV-1 have shown that the cytoplasmic tail plays an important role in virus infectivity; truncation of the cytoplasmic tail C terminus by 16 amino acids severely impaired virus infectivity (8, 10, 22). Previous studies have reported that the cytoplasmic tails of SIV and HIV envelope proteins could affect their fusion activity, since envelope proteins with truncated cytoplasmic tails acquired increased syncytium-forming ability (26, 32, 36, 43). The best-studied example of this effect is SIV, which has a naturally truncated variant that is infectious (5, 16). In other retroviruses, including MuLV, Mason-Pfizer monkey virus, and equine infectious anemia virus, the envelope protein cytoplasmic tail is further processed by the viral protease during virus maturation (6, 11, 31, 36, 37). Recent studies have reported that cleavage of the R peptide of MuLV envelope protein is important to activate its fusion activity (28, 30). However, it was reported that fusion with XC cells was not affected significantly by truncation of the R peptide (17, 29). Under the conditions used in the present study, the R peptide was also found to modulate the fusion activity of the MuLV envelope protein with XC cells. Denesvre et al. (7) recently reported that a chimeric envelope protein with a human T-cell lymphophotrophic virus type I external domain and the MuLV transmembrane domain and cytoplasmic tail was able to induce fusion in various cell lines, including XC cells. However, the chimeric envelope protein used in their studies did not contain the full-length MuLV cytoplasmic tail but a molecule truncated in the last seven amino acids. This highly hydrophilic
Further understanding of the function played by the R peptide preventing this segment from interacting with cellular factors, features, including the ability to form amphipathic helixes. The cytoplasmic tails of the naturally truncated R peptide does not inhibit the cell fusion activity by a partially unaffected (data not shown), indicating that the R peptide expressed with R peptide-containing envelope proteins, the cell fusion activity either by association with cellular proteins that could inhibit fusion or by preventing the envelope proteins from interacting with the cellular proteins that promote cell fusion. When R peptide-truncated envelope proteins were coex- pressed with R peptide-containing envelope proteins, the cell fusion activity of the truncated envelope proteins was essentially unaffected (data not shown), indicating that the R peptide does not inhibit the cell fusion activity by a trans-dominant mechanism. The cytoplasmic tails of the naturally truncated SIV envelope protein and the processed MuLV envelope protein are similar in size, and they exhibit some similar structural features, including the ability to form amphiphilic helices. Possibly, the R peptide could exert its inhibitory effect by preventing this segment from interacting with cellular factors. Further understanding of the function played by the R peptide in inhibiting fusion activity should yield more information on the mechanism of cell fusion and the possible involvement of cellular factors in the cell fusion process.

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